ECOLOGY AND THERMAL INACTIVATION OF MICROBES IN AND ON INTERPLANETARY SPACE VEHICLE COMPONENTS

First Quarterly Report of Progress

on

Research Project R-36-015-001

April 1 - June 30, 1965

Conducted by

Milk and Food Research, SEC
Division of Environmental Engineering
and Food Protection

for the

National Aeronautics and Space Administration
Washington, D. C.

U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
Public Health Service
Division of Environmental Engineering and Food Protection
Robert A. Taft Sanitary Engineering Center
Cincinnati, Ohio
July, 1965

ECOLOGY AND THERMAL INACTIVATION OF MICROBES IN AND ON INTERPLANETARY SPACE VEHICLE COMPONENTS

First Quarterly Report of Progress

on

Research Project R-36-015-001

April 1 - June 30, 1965

CONTRIBUTORS

Food Microbiology

Robert Angelotti
Herbert E. Hall
James H. Maryanski
Ronald G. Crawford

Milk Sanitation

Ralston B. Read

Food Chemistry

J. E. Campbell
James E. Gilchrist

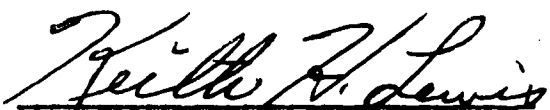
Statistics

James T. Peeler

Report Submitted by:

Robert Angelotti
Microbiologist

Report Reviewed and Forwarded by:



Keith H. Lewis
Principal Investigator

CONTENTS

SUMMARY

Several batches of Bacillus globigii spores have been produced with an average count of approximately 2×10^9 spores per ml after heat shocking at 80° C for 10 minutes. Several plating media were compared to determine their ability to permit germination and good outgrowth of B. globigii spores. Tryptone glucose extract agar was selected as the medium of choice as a result of these comparisons.

Prototype models of two pieces of apparatus designed to reduce solids to particle sizes in the range of 5 to 50 microns have been developed, and their operational characteristics are being evaluated to determine their compatability with microbiological requirements. As a part of these evaluations, the effects of acetone, silicon carbide grinding papers and powders, and aluminum oxide grinding powders on the growth of Bacillus globigii spores was determined. Silicon carbide grinding paper (grit size 320) exhibited some inhibitory properties whereas similar papers of grit sizes 220 and 400 did not. No inhibition of growth was noted after storage of B. globigii spores in acetone for 72 hours or when they were plated in media made with water that had been used to leach silicon carbide and aluminum oxide powders.

Author

ECOLOGY AND THERMAL INACTIVATION OF MICROBES IN AND ON INTERPLANETARY SPACE VEHICLE COMPONENTS

I. Introduction

Space vehicle component parts are contaminated with microbial forms both in their interiors and on their external surfaces. Phillips and Hoffman (1960) and Koesterer (1965) reported on the natural internal contamination of electronic components used in the fabrication of space vehicles. In order to prevent contamination of other planets with microbial forms of earth origin, methods need to be developed to sterilize the surfaces and internal structures of space vehicles. Dry heat sterilization as a terminal treatment prior to blast-off appears to be feasible and exposure temperatures of 135° C or less are compatible with maintaining functional properties of component parts (L. B. Hall, 1965, Personal Communication).

The dry heat inactivation of spores of various aerobic and anaerobic bacterial species incorporated in or on paper, sand, glass, solid rocket propellents, asbestos, plaster of Paris and dental compound has been investigated by Bruch et al., (1963). These studies indicate that the D values for these spores range from a few minutes at exposure temperatures of 160° C to more than six hours at 120° C depending upon the bacterial species and carrier involved. Koesterer (1965) reported consistent killing of large concentrations of spores placed in or on a number of carriers by exposure to 135° C for 24 hours. He also found that the natural surface contamination of objects exposed to airborne microbial spore fallout in dirty environments could be sterilized at 275° F within a few hours.

Due to the limited amount of information available concerning the dry heat processes required to destroy spores in or on various materials, it appears desirable to collect specific information on the dry-heat resistance of spores associated with space vehicle components. In addition, techniques are needed through which it is possible to quantitatively detect small numbers of spores contained within the interior structure of various materials used in space craft fabrication. Because of the strict statistical limitation (no more than one spore in every 10,000 samples) placed upon the terminal sterilization process it will be necessary to determine experimentally the efficacy of the process rather than to depend upon extrapolations of existing data. To do the former requires that methods be developed by which known numbers of spores can be incorporated into materials and quantitatively recovered. Following this, these methods can then be applied to experimentally determine that certain processes truly sterilize within known statistical limits of confidence and that negative recovery data do not, in fact, merely indicate an inability to recover the few surviving spores.

It is toward the above goals that this project is directed and efforts will be expended in determining survival curve end points (D values) of Bacillus globigii spores in various materials at dry heat temperatures of 105, 115, 125 and 135° C. A significant portion of the contract effort will also be directed toward developing methods for the quantitative and reproducible recovery of B. globigii spores from plastics, potting compounds and similar materials. A statistical

level of assurance will also be determined that no more than one spore in every 10,000 samples will survive each time-temperature process.

II. Experimental

Equipment and personnel.

Funding of the contract commenced April 12, 1965 and immediately thereafter purchase requisitions were submitted for equipping the special laboratory in which the major share of the contract work is being performed. The major pieces of equipment ordered included: a Freas mechanical convection dry heat oven capable of maintaining a uniform temperature throughout the box ($< \pm 1^\circ$ C) and with a sensitivity of $\pm 0.5^\circ$ C; a four glove-port all plastic isolator hood for performing sterility studies; equipment for reducing solids to small particles including an air abrasive impinger, micro-lathe, and a unit for smashing solids frozen in liquid nitrogen.

In addition to the purchase of these specialized pieces of equipment, adequate quantities of conventional laboratory supplies were ordered consisting of glassware, media, reagents and utensils.

Recruitment of personnel is still in progress and it is anticipated that the remaining vacancy for a bacteriologist will be filled shortly.

Microbiological procedures.

Spores of Bacillus globigii (BG) were received from the U. S. Army Biological Research Center, Fort Detrick, Maryland. Aliquots of these spore preparations were surface plated on tryptone glucose beef extract (TGE) agar to obtain isolated colonies and incubated at 35° C. Portions

of typical rust colored colonies were again similarly streaked and incubated and portions of well isolated colonies were streaked to TGE slants and incubated at 35° C until heavy growth was apparent. These slant cultures were studied for biochemical reactions in accordance with Bergey's Manual of Determinative Bacteriology and those cultures meeting the criteria described for B. globigii, including typical pigment formation on solid media as well as black pigment formation on tyrosine containing media, were reserved as parent stocks from which spore crops would be produced.

Six crops of spores have been produced to date by surface inoculation of spore growth agar medium (Seitz filtered glucose, 0.25%; casamino acids (tech.), 0.25%; yeast extract, 0.5%; $MnSO_4 \cdot H_2O$, 0.001%; $FeSO_4 \cdot 7H_2O$, 0.0014%) in six liter Pavitsky bottles followed by incubation for seven days at 35° C. Spores are harvested by adding approximately 150 ml of double distilled sterile water to the bottle and gently scraping the surface mat of growth to obtain a heavy suspension of spores. The spore suspension is shaken with glass heads and then filtered through sterile non-absorbent cotton. Additional double distilled sterile water is passed through the cotton to make a final volume of approximately 400 ml. The spore suspension is centrifuged at 5000 rpm for 30 minutes in the cold (5° C). The sediment is resuspended in fresh double distilled sterile water (400 ml) and placed in a 45° C water bath overnight. Following the heat treatment, the suspension is centrifuged and washed five additional times in 400 ml of double distilled sterile water at 5000 rpm for 20 minutes at 5° C.

The final spore suspension is observed microscopically to determine freedom from cellular debris (Bartholmelu - Mittwer stain) and the clean suspensions are stored at 5° C. The number of spores per ml is determined at weekly intervals as follows: aliquots of the clean spore crops are heat-shocked at 80° C for 10 minutes and serial ten-fold dilutions are prepared in phosphate buffered dilution water (APHA) and plated in triplicate in TGE agar. The plates are incubated for 48 hours at 35° C and counted. Plate counts of aliquots of the non-heat shocked spore suspensions are additionally prepared using the same procedures.

A comparison was made in a number of solid plating media of the ability of heat-shocked (80° C for 10 minutes) and non-heat-shocked BG spores to germinate and produce visible colonies. The media compared were: plate count agar; TGE agar; tryptose agar + 0.005% thiamine HCl; nutrient agar + 0.5% yeast extract. With the exception of tryptose agar all media were additionally prepared to contain 0.2% soluble starch. An aliquot of stock spore suspension was heat shocked and ten-fold serial dilutions were prepared in phosphate buffered dilution water. Triplicate one-ml aliquots of each of the dilutions were plated in each of the above media. The inoculated plates were incubated at 35° C for 48 hours. A non-heat-shocked aliquot of the same spore suspension was diluted, plated and incubated in like fashion.

The effect of acetone on the viability and germination of heat-shocked and non-heat-shocked BG spores was studied. Duplicate aliquots of the aqueous stock spore suspensions stored in the cold were removed, and one aliquot was heat shocked at 80° C for 10 minutes. Following this treatment, three ml of the heat shocked suspension were placed in

27 ml of acetone and a second three-ml aliquot was placed in 27 ml of phosphate buffered dilution water. Additional ten-fold serial dilutions of the acetone suspension were prepared in acetone, whereas additional ten-fold serial dilutions of the buffered water suspension were prepared in buffered water. Three-ml aliquots of the non-heat-shocked stock spore suspension were similarly diluted in acetone and phosphate buffered dilution water. Immediately after preparing all the dilutions 1-ml aliquots of each were plated in triplicate in TGE agar and the plates were incubated for 48 hours at 35° C. The acetone and buffered water suspensions of spores were held at room temperature for 72 hours and again were plated in triplicate at 24 hour intervals.

A study was made to determine whether various commercially available grinding papers and powders exerted any inhibitory effects upon the germination and growth of BG spores. Silicon carbide and aluminum oxide grinding powders in the particle size range of 10 to 50 microns were examined as well as silicon carbide grinding papers of grit sizes 220, 320 and 400.

The powders were sterilized by dry heating at 158° C for 3 hours. Fifty grams of each of the sterile powders were placed in 200 ml of distilled, sterile water and allowed to leach for 1 week at room temperature. Following leaching, the powder suspensions were centrifuged at 5000 rpm for 20 minutes and the supernatent water was collected aseptically and used to reconstitute to single strength perviously sterilized TGE agar made up to 4 X concentration. One-tenth ml aliquots of heat-shocked (80° C for 10 min.) stock spore suspension diluted to contain approximately 100 to 200 spores per 0.1 ml were plated in triplicate and poured

with the TGE agar. The plates were incubated 48 hours at 35° C.

The grinding papers were cut into 2 x 2 inch squares. A square of paper was placed in 100 ml of distilled water and leached by autoclaving at 121° C for 15 minutes. Following leaching, the paper was removed and rinsed in 200 ml of distilled water. The square was then placed in 100 ml of melted but not sterile TGE agar and autoclaved at 121° C for 15 minutes. After cooling, the agar was used to pour triplicate plates inoculated with 0.1-ml aliquots of heat-shocked (80° C for 10 min.) spore suspension diluted to contain approximately 100 to 200 spores per 0.1 ml. The plates were incubated at 35° C for 48 hours. As controls, non-leached 2 x 2 inch squares of paper were placed in 100 ml of melted but not sterile TGE agar and the medium was sterilized as above. Triplicate plates of the same heat-shocked spore suspension were poured with this medium and similarly incubated.

Procedures for disintegrating solids to small particles.

A mathematical model was developed to consider problems related to the contamination solids. It was assumed, for simplicity, that the bacteria are uniformly distributed within a solid cylinder of length l and radius r. It was also assumed that if a slice 1μ thick is made perpendicular to the length, that all the organisms in this volume would grow when the slice was placed in an appropriate medium. The percent of the total number of organisms in this slice is $P = \frac{1\mu \pi r^2}{l \pi r^2 h} 100 = \frac{100\mu}{l}$. Consider a rod of 1 meter. The percent of the total organisms expected in the slice is $P = 100 / 10^6 = 0.0001$ percent. In order to get P = 50% the area would be $(2 \times \pi r^2)(5,000)$. It is obvious that a large amount of area must be generated in order to measure a large percentage of organisms. Accordingly, systems for reducing solids to small particle

size are needed in order to obtain sufficient surface area for quantitatively recovering microorganisms from the internal structure of components. Two systems of reducing solids to small particle size of predictable dimensions in the 5-50μ range are being developed. The first system consists of a wet grinding process in which the component is ground in a liquid recovery medium contained in a modified Waring Blendor cup in which the knives have been replaced by grinding disks. The liquid system is kept cool during the grinding process by means of a cooling coil. The second system consists of an abrasive impinging technique based on the use of an industrial air abrasive unit operated under liquid recovery media.

Prototype models of both these systems have been developed and design modifications are under study in order that their final operating characteristics are compatible with microbiological requirements.

III. Results

All the spore crops produced to date have yielded spore concentrations in the range of 1×10^9 to 2×10^9 per ml after heat-shocking at 80° C for 10 minutes. No significant changes in spore populations have been noted over a period of several weeks storage at 5° C.

Studies on the germination and outgrowth of separate crops of BG spores in several plating media revealed that, with the exception of tryptose agar, all the media yielded equivalent results. Tryptose agar yielded slightly lower recovery values (See Table I). In view of these data, TGE agar was selected as the medium of choice for future experiments concerning germination and outgrowth.

No appreciable differences were noted between the effects of acetone

and buffered dilution water on the germination and outgrowth of BG spores after 72 hours storage in both liquids (See Table II).

The silicon carbide and aluminum oxide grinding powders tested did not adversely affect germination or outgrowth of BG spores, whereas, commercially available silicon carbide grinding paper of grit size 320 appeared to be slightly inhibitory even after leaching (See Tables III and IV).

The prototype grinding models have been employed to fragment various forms of plastic and through the proper combination of grinding powders or grit size papers particles in the 5 to 50 micron range have been consistently obtained.

IV. Projected Efforts for Second Quarter

Activities during the next quarter will be directed toward (a) devising a system of minimizing uneven distribution of temperature and excessive heat losses in dry-air ovens; (b) undertaking thermal-death-time studies on BG spores on surfaces; (c) initiating studies on methods for the incorporation and quantitative recovery of spores in plastic.

V. References

1. Bruch, C.W., Koesterer, M.G., and Bruch, M.K., 1963 Developments in Industrial Microbiology, Vol. 4, 334-342.
2. Koesterer, M.G., 1965 Studies for sterilization of space probe components. NASA Contractor Report 191, National Aeronautics and Space Administration, Washington, D. C., March, 1965.
3. Phillips, C.R. and Hoffman, R.K. 1960 Sterilization of Interplanetary Vehicles, Science, Vol. 132, No. 3433, 991-995.

Table I

Growth of Bacillus globigii Spores in Various Plating Media After Incubation at 35° C for 48 hours

Spore Crop	Plating Media	Heat Shocked* No. of spores per ml 1×10^{9}**	Non-heated No. of spores per ml 1×10^{9}**
A	Plate Count agar	1.1	1.2
	Plate Count agar + 0.2% soluble starch	1.0	1.3
	Spore germination agar	1.0	1.0
	Spore germination agar + 0.2% soluble starch	1.1	1.3
	Tryptose glucose extract agar	1.2	1.3
	Tryptose glucose extract agar plus 0.2% soluble starch	1.1	1.4
B	Tryptose agar	0.6	1.0
	Tryptose glucose extract agar	0.9	4.0

* Heat-shocked at 80° C for 10 minutes

**Mean of triplicate plate count values

Table II

Growth in Tryptone Glucose Extract Agar of Bacillus globigii Spores After Storage in Acetone and Phosphate Buffered Dilution Water

Suspending medium	Hours of incubation	Heat-shocked*	Non-heated
		No. spores recovered per ml (1×10^9)**	No. spores recovered per ml (1×10^9)**
Acetone	0	2.5	2.8
	24	2.5	2.5
	48	2.6	2.7
	72	2.5	2.6
Phosphate Buffered Dilution Water	0	2.5	2.6
	24	2.5	2.8
	48	2.5	2.7
	72	2.5	2.6

* Heat-shocked for 10 min. at 80° C.
** Mean of triplicate plate count values. Incubation for 48 hours at 35° C.

Table III

Toxicity of Aqueous Leachings of Aluminum Oxide and Silicon Carbide Grinding Powders on *Bacillus globigii* spores

TGE agar prepared with:	Number of spores recovered* (Heat-shocked at 80° C for 10 min.)
Distilled water	138
Leach water from aluminum oxide powders of particle sizes:	
10μ	137
25μ	137
50μ	124
Leach water from silicon carbide powders of particle sizes:	
10μ	137
25μ	138
50μ	130

*Mean of triplicate plate count values. Incubation 48 hours at 35° C.

Table IV

Toxicity of Commercially Available Silicon Carbide Grinding Papers on *Bacillus globigii* spores

Plating Media	Number of spores recovered* (Heat-shocked 80° C for 10 min.)
TGE agar control	173
100 ml TGE + 4 sq. in. of paper grit size 220	189
100 ml TGE + 4 sq. in. of paper grit size 320	146
100 ml of TGE + 4 sq. in of paper grit size 400	178
100 ml TGE + 4 sq. in. of leached and rinsed paper grit size 220	191
100 ml of TGE + 4 sq. of leached and rinsed paper grit size 320	147
100 ml of TGE + 4 sq. in. of leached and rinsed paper grit size 400	178

*Mean of triplicate plate count values. Incubation 48 hours at 35° C.